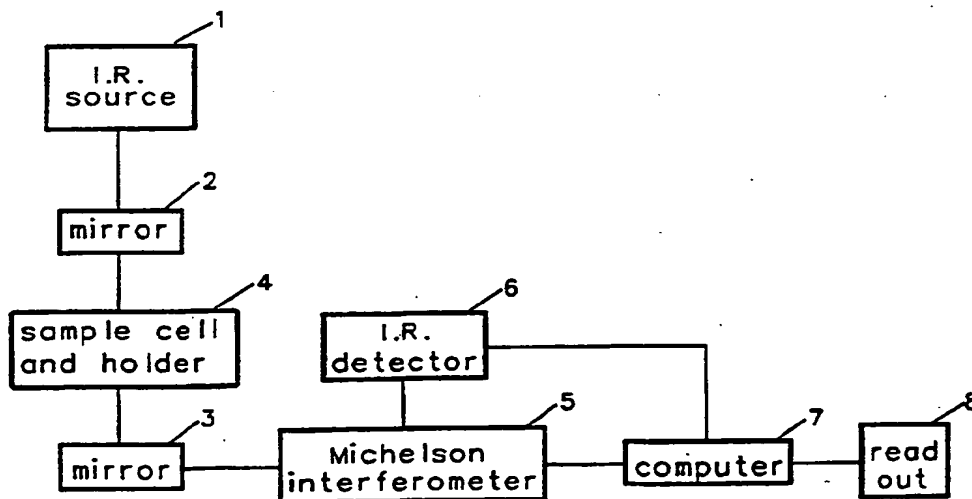




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : G01N 21/35, 33/483, C12M 1/34		A1	(11) International Publication Number: WO 96/00892
			(43) International Publication Date: 11 January 1996 (11.01.96)
(21) International Application Number: PCT/CA95/00370		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 23 June 1995 (23.06.95)			
(30) Priority Data: 2,126,915 28 June 1994 (28.06.94) CA 08/401,442 9 March 1995 (09.03.95) US			
(71)(72) Applicants and Inventors: WONG, Patrick, T., T. [CA/CA]; 35 MacNabb Place, Ottawa, Ontario K1L 8J5 (CA). WONG, Rita, K. [CA/CA]; 35 MacNabb Place, Ottawa, Ontario K1L 8J5 (CA).			
(74) Agent: LEMON, Francis, W.; 67 First Avenue, Brockville, Ontario K6V 3A9 (CA).		Published With international search report.	

(54) Title: INFRARED SPECTROSCOPY OF A SAMPLE TREATED WITH PRESERVATIVE



## (57) Abstract

The presence of anomalies in biological tissue and cells in natural or cultured form (e.g. cancerous tissue or cells) is detected by directing a beam of infrared light at a preservative treated sample, prepared from fresh biological tissue or cells, in either the transmission mode or the Attenuated Total Reflectance (ATR) mode. The anomaly is then determined by whether changes in infrared absorption have occurred, due to the vibration of at least one functional group of molecules present in the sample, which are characteristic of the anomaly. The preservative may be an aqueous solution of formalin, an aqueous solution of ethyl alcohol, or an aqueous solution of inorganic salts. The preservative treatment prevents degradation of the sample at room temperature which has been found to change the infrared absorption characteristics which can lead to misinterpretations.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## INFRARED SPECTROSCOPY OF A SAMPLE TREATED WITH PRESERVATIVE

5

This invention relates to a method of detecting the presence of anomalies in a preservative treated sample, prepared from fresh biological tissue or cells in natural or cultured form, by infrared spectroscopy.

10 **BACKGROUND TO THE INVENTION**

In an attempt to detect the presence of leukemia, it has already been proposed by E. Benedetti et al in Leukemia Research, pp. 1001-1008, vol. 9, Nov. 8, 1985 to,

- 15 i) chemically separate lymphocytes from other cells and components in blood,  
ii) vacuum dry the separated lymphocytes,  
iii) mix the dried lymphocytes with powdered KBr as a binder,  
iv) grind the lymphocytes/KBr mixture to a fine powder,  
20 v) compress the fine powder into a pellet in a sample holder, and  
vii) measure the infrared spectra of the sample by infrared spectrometer.

While the process of Benedetti et al is useful, the cellular structure is completely destroyed by it, resulting in the loss of important information from  
25 which tissue or cell anomalies can be deduced. Furthermore, Benedetti et al make no provisions to preserve the lymphocyte cells against deterioration. These drawbacks severely restrict the uses to which the Benedetti et al process can be put.

It has also been proposed in the United States Patent No. 5,038,039, dated August 6, 1991, P. T. T. Wong et al, to detect the presence of anomalies in biological tissue or cells in natural and cultured form by infrared spectroscopy. In this process, a sample of biological tissue or cells as they occur in nature, or as they may be cultured, which have been dispersed in water, or sliced, but which otherwise remain in natural form, has a beam of infrared light directed thereon. While this is occurring, spectroscopic analysis is used to determine whether infrared absorption has occurred, at least one range of frequencies, due to vibration of at least one functional group of molecules present in the sample which is a characteristic of the anomaly.

The method of P.T.T. Wong et al, 5,038,039, is particularly useful in detecting malignancy in human colon epithelial cells, colon tumor tissue and liver tumor tissue.

United States patent No. 5,168,162 dated December 1, 1992, P.T.T. Wong et al, extends the method of P.T.T. Wong et al, 5,038,039 to detecting the presence of anomalies in exfoliated cells, and in this instance is particularly useful in detecting malignancy in cervical cells obtained by Papanicolaou smear.

While the methods of detection of P.T.T. Wong et al, 5,038,039 and 5,168,162 have proved to be useful, the specimens have had to be frozen in order to avoid degradation. Even for a short time period, at room temperature it has now been found that significant changes in spectral absorption will occur. While the increase of lipids containing branched fatty acids, for period of five hours or longer, in thymus tissue is disclosed in P.T.T. Wong et al, 5,038,039 (see column 8, lines 21 to 32), this patent would not lead a person skilled in the art to believe that changes in spectral absorption occur at room temperature,

even for very short time periods, in tissue or cells, that are of sufficient magnitude to give such misleading readings, that in some instances, anomalies are presumed to be present where they do not exist. Even more significant is the surprising finding by the applicants that these changes in spectral absorption can  
5 give readouts similar to those of, for example, cancerous tissue or cells.

Conventionally, when a surgical specimen is obtained for histological examination, it is frozen to be preserved during transportation to the pathology laboratory where the histological examination will be carried out. On arrival at  
10 the laboratory, the specimen is sliced by frozen microtome sectioning techniques to provide the tissue sample for the histological examination.

For examination by infrared spectroscopy in accordance with United States Patent No. 5,038,039, it is necessary for the sample thickness to be less  
15 than about 20 microns and examined after being thawed at room temperature.

If the sample is allowed to thaw and remain at room temperature for even a short period of time, it has now been found that degradation can occur which may change the infrared absorption and, in some instances, give a misleading  
20 reading so that malignancy is presumed where it does not exist.

It has already been proposed in Chemical Infrared Spectroscopy, W. J. Potts Jr., Volume I, Technique, 1963, pages 238-242, to study the optical properties, especially infrared intensities of chemical materials, e.g. crosslinked  
25 polymers and resins, by filling a cavity in a small, solid, cell block of sodium chloride, masking off the cell so that light falling on its outer surface can only pass through the small cell volume, and then mounting the cell in an infrared beam-condensing system. An infrared spectrum may be obtained by reflection

of radiation from the surface of the chemical material (rather than by transmission through it) usually produces a spectrum that was so poor as to have little value. However, a technique known as attenuated total reflection, often abbreviated ATR, had recently been conceived, when this article was written, whereby reflection spectra of a much more satisfactory quality was obtained.

While the process taught by W. J. Potts, Jr., is useful for virtually indestructible organic polymeric and resinous materials, it would not lead a person skilled in the art to use sodium chloride to preserve room temperature degradable samples, such as samples prepared from fresh biological tissue or cells, for the detection of anomalies therein by infrared spectroscopy. Further, water present in such samples would dissolve the sodium chloride cell rendering it useless. However, W. J. Potts, Jr., does show that it was known to obtain an infrared spectrum by reflection of radiation from a sample, or by transmission through it, at least as early as 1963.

In "Applied Infrared Spectroscopy, Fundamentals, Techniques, and Analytical Problem-solving", A. Lee Smith, 1979, pages 84-86, it is taught on page 86 that the reflected radiation of ATR spectroscopy could yield an absorption that closely resembled that of a transmission spectrum.

There is a need for a method of detecting the presence of anomalies in biological tissue or cells in natural or cultured form, by infrared spectroscopy wherein room temperature degradation of the tissue or cells is retarded to the extent that it does not interfere with the detection of the anomaly or give a misleading readout.

## SUMMARY OF THE INVENTION

According to the present invention, there is provided a method of detecting the presence of anomalies in a preservative treated sample, prepared from fresh biological tissue or cells in natural or cultured form, by infrared spectroscopy, comprising:

- a) directing a beam of infrared light at the treated sample, and
- b) determining, by spectral analysis, at least one range of frequencies, whether variation in infrared absorption occurs in the treated sample, distinguishable from any variation therein resulting from the treatment, due to vibration of at least one functional group of molecules that are present in the treated sample and characteristic of an anomaly.

The determination by spectral analysis may comprise comparing infrared spectra, obtained by directing the beam of infrared light at the treated sample, and spectral parameters derived therefrom, at the said at least one range of frequencies, with similar infrared spectra, and spectral parameters derived therefrom, from a similar sample, treated in the same manner, and known to have been prepared from fresh, normal, healthy tissue or cells.

If, by this comparison, no variation in infrared spectra, or spectral parameters obtained therefrom, is apparent between the sample and the control sample, then clearly the determination by spectral analysis is complete in that it has revealed that an anomaly is not present in the sample and the sample is healthy and normal.

If, however, variation in infrared spectra, or spectral parameters obtained therefrom, is apparent between the sample and the control sample, then clearly

further comparison is desirable to determine just what the anomaly is that is causing the variation.

Further, according to the present invention, the determination by spectral analysis comprises, comparing infrared spectra obtained by directing the beam of infrared light at the treated sample, and spectral parameters derived from the spectra, at the said at least one range of frequencies, with similar infrared spectra, and spectral parameters derived therefrom, from at least one similar sample, treated in the same manner, and having a known anomaly.

10

It is within the scope of the present invention to merely test a treated sample for at least one particular anomaly, without testing the sample by comparison with treated normal, healthy tissue or cells.

15

The determination by spectral analysis may be obtained from infrared radiation from the beam that has passed through the treated sample, at the said at least one range of frequencies.

20

The determination by spectral analysis may be obtained from attenuated, totally reflected infrared radiation of the beam from the sample at the said at least one range of frequencies.

25

The preservative treated sample may be a sample that has been treated with a preservative selected from the group consisting of formaldehyde, alcohols, salines and inorganic salts.

The formaldehyde may be an aqueous solution of formalin.



The alcohol may be methyl alcohol, ethyl alcohol or their mixture.

The saline may be normal saline, phosphate buffered saline or a physiologically balanced salt solution consisting of calcium chloride, magnesium chloride, potassium chloride, sodium acetate, sodium chloride, and sodium citrate.

The inorganic salt may be sodium nitrate, sodium chloride or potassium bromide.

The said at least one range of frequencies may be at least one frequency in at least one range of frequencies within the ranges  $500\text{ cm}^{-1}$  to  $900\text{ cm}^{-1}$ ,  $900\text{ cm}^{-1}$  to  $1500\text{ cm}^{-1}$ ,  $900\text{ cm}^{-1}$  to  $1800\text{ cm}^{-1}$  and  $2000\text{ cm}^{-1}$  to  $6000\text{ cm}^{-1}$ .

In this specification, fresh biological tissue or cells in natural or cultured form means biological tissue or cells newly obtained, cultured tissue or cells, or cells exfoliated from fresh biological tissues which are substantially free of degradation by exposure to room temperature.

Surprisingly, tests have shown that anomalies in preservative treated samples from freshly obtained biological tissue or cells in natural or cultured form can be detected by infrared spectroscopy, even though some preservatives have been found to cause slight changes in the spectra. Put another way, the preservatives have been found not to mask infrared absorption due to the anomalies or give misleading readouts. What is essential is that the infrared spectra be analyzed differently. It is particular important that the infrared spectra, and the spectral parameters derived therefrom, from the sample, be compared with the infrared spectra, and the spectral parameters derived

therefrom, from a similar sample, treated in the same manner, and known to be either normal and healthy or to have a known anomaly.

5 The biological tissue or cells may be obtained from various human or other mammalian organs or tissues.

The said at least one change in infrared absorption characteristic may be a change in absorption intensity at a particular frequency region, a change of frequency at which a particular absorption occurs, or a different pressure applied to the functional group causing a change of frequency at which a particular  
10 absorption occurs.

As taught in United States Patent No. 5,168,162, dated December 1, 1992, the functional group of molecules may be in at least one of the following  
15 molecules, carbohydrates, nucleic acids, tissue proteins, or membrane lipids.

Also, as taught in United States Patent No. 5,168,162, dated December 1, 1992, the functional group may be a phosphodiester group in nucleic acids, a C-OH group in tissue proteins and carbohydrates, a CH<sub>2</sub> group in lipids, or CH<sub>3</sub>  
20 groups in proteins, nucleic acids and lipids..

Also, as taught in United States Patent No. 5,168,162, dated December 1, 1992, the functional group may be at least one functional group selected from the groups consisting of CH<sub>2</sub>-OH group in carbohydrates, phosphodiester  
25 groups in nucleic acids, C-OH groups of tissue proteins and carbohydrates, CH<sub>2</sub> groups of lipids, and CH<sub>3</sub> groups in proteins, nucleic acids and lipids.

However, for some preservatives, because of the presence of the preservative, the spectra will be slightly different to those given in United States Patent No. 5,168,162, dated December 1, 1992.

5 The specimens may be prepared from fresh

- a) microtome sections of tissue biopsy,
- b) punched or needle tissue biopsy,
- c) cultured cells, or
- d) exfoliated cells such as, for example,

- 10 i) Papanicolaou smears,
- ii) cervical specimens,
- iii) endocervical specimens,
- iv) ectocervical specimens,
- v) vaginal specimens,
- 15 vi) uterus specimens, or
- vii) bronchial specimens.

When the specimen is tissue it may be liver tissue, and the anomaly an indication of malignancy in the liver tissue.

20

When the specimen comprises cells, the cells may be human ovarian epithelial cells.

When the specimen is tissue, the tissue may be cervical tumor tissue and  
25 the anomaly an indication of malignancy in the tissue.

When the specimen is exfoliated cells, the specimen may be obtained from scraping, brushing, washing, secretions, exudates or transudates from various organs and tissues.

5 With specimens obtained in any manner, the anomaly may also indicate the presence of, for example, precancerous lesions, viruses, bacteria, fungi, and other infectious or non-infectious diseases.

### BRIEF DESCRIPTION OF THE DRAWINGS

10 In the accompanying drawings which illustrate, by way of example, embodiments of the present invention,

Figure 1 is a block diagram of an apparatus for detecting the presence of  
15 anomalies in a preservative treated sample, prepared from fresh biological tissue or cells in natural or cultured form using infrared spectroscopy,

Figure 2 shows infrared spectra in the frequency range 2800 to 3020  $\text{cm}^{-1}$   
1 obtained from a fresh, normal, healthy, liver tissue specimen that was not  
20 treated with a preservative and after keeping the specimen at room temperature and measured after the passage of various time periods,

Figure 3 shows similar infrared spectra to that shown in Figure 2, but for  
normal healthy and malignant tissue not treated with a preservative,

25 Figure 4. shows infrared spectra in the frequency range 900 to 1500  $\text{cm}^{-1}$  obtained from an air dried specimen of fresh, exfoliated cervical cells which has

been diagnosed as high grade dysplasia and was not treated with a preservative, and after keeping the specimen at room temperature for three days,

5 Figure 5. shows infrared spectra in the frequency range 900 to 1500  $\text{cm}^{-1}$  obtained from a preservative treated wet specimen of fresh, normal, healthy, exfoliated cervical cells and after the wet specimen was air dried at room temperature on a sample cell with a single infrared window,

10 Figure 6 shows infrared spectra in the frequency range 900 to 1800  $\text{cm}^{-1}$  obtained from a preservative treated and air dried specimen of fresh, normal, healthy, exfoliated cervical cells and after keeping the specimen at room temperature for three months,

15 Figure 7 shows infrared spectra in the frequency range 900 to 1800  $\text{cm}^{-1}$  obtained from a preservative treated and air dried specimen of fresh, exfoliated cervical cells which has been diagnosed as low grade dysplasia (CIN 1), and after keeping the specimen at room temperature for three months,

20 Figure 8 shows infrared spectra in the frequency range 950 to 1500  $\text{cm}^{-1}$  obtained from a preservative treated specimen of fresh, normal, healthy, exfoliated cervical cells and after keeping the specimen at room temperature for 15 hours,

25 Figure 9 shows infrared spectra in the frequency region 950 to 1500  $\text{cm}^{-1}$  obtained from a preservative treated specimen of fresh, exfoliated cervical cells which has been diagnosed as moderate dysplasia (CIN II), and after keeping the specimen at room temperature for one and half months,

Figure 10 shows infrared spectra in the frequency range 950 to 1500  $\text{cm}^{-1}$  obtained from two samples of preservative treated biopsy of cervical tissue, prepared from fresh tissue, one of which is normal healthy tissue and the other of which has been diagnosed in a conventional, histological manner as malignant,

Figure 11 shows infrared spectra in the frequency range 950 to 1500  $\text{cm}^{-1}$  obtained from two samples of preservative treated, exfoliated cervical cell specimens from fresh cells, one batch of which is normal healthy cells and the other batch of which has been diagnosed as inflammatory,

Figure 12 shows infrared spectra in the frequency range 900 to 1500  $\text{cm}^{-1}$  obtained from two samples of preservative treated, air dried, exfoliated cervical cell specimens from fresh cells, one batch of which is normal, healthy cells and the other batch of which has been diagnosed as having Candida infection,

Figure 13 shows infrared spectra in the frequency range 950 to 1500  $\text{cm}^{-1}$  obtained from two samples of preservative treated, exfoliated cervical cell specimens from fresh cells, one batch of which is normal healthy cells and the other batch of which has been diagnosed as having dysplasia, CIN 1, HPV (human paparoma virus), and

Figure 14 shows ATR infrared spectra in the frequency range 950 to 1500  $\text{cm}^{-1}$  obtained from a preservative treated and air dried specimen of cultured malignant ovarian cells, and keeping the specimen at room temperature for 20 hours.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

Referring now to Figure 1, there is shown an infrared source 1, infrared beam focusing mirrors 2 and 3, a sample cell and holder 4, a Michelson interferometer 5, a infrared light detector 6, a computer 7, and a readout 8. In the following tests, the infrared source 1, infrared beam focusing mirrors 2 and 3, the holder of the sample cell and holder 4, Michelson interferometer 5, and infrared light detector 6 were components of a Nicolet Magna IR 550 Fourier-transform infrared spectrometer obtainable from Thermo Instruments (Canada) Inc., Mississauga, Ontario, Canada. The sample cell of the sample cell and holder 4 for measuring the transmission infrared spectra had a single infrared optical window on which the specimen was deposited or two infrared optical windows between which the specimen was placed. The sample cell of the sample cell and holder 4 for measuring ATR infrared spectra was a contact Sampler<sup>TM</sup> ATR accessory (Spectra-Tech, Inc.) with a ZnSe crystal, obtained from Spectra-Tech, Inc., Stamford, Connecticut.

In operation, a biological tissue or cell sample was placed in a transmission or ATR sample cell of the sample cell and holder 4, a beam of infrared light from the source 1, which had been condensed by the focusing mirror 2, was passed through the sample in the sample cell and holder 4 and was focused to the detector 6 through the Michelson interferometer 5 by the focusing mirror 3. Any infrared absorption by an anomaly in the specimen was detected by the Michelson interferometer 5 and the detector 6, which, in turn, was computed by the computer 7 to give a readout at the readout 8. The computer readout may be programmed for the readout 8 to directly indicate whether the sample is a normal healthy one or one which contains an anomaly, which may be, for example, benign, dysplasia, or malignant.

In the following tests to verify the present invention, infrared spectra were obtained using the apparatus described with reference to Figure 1 from samples of tissue biopsies, scraping tissue to obtain exfoliated cells and cultured  
5 cells.

In Figure 2, infrared spectra are shown that were obtained in the range 2800 to 3000  $\text{cm}^{-1}$  from a fresh, normal, healthy liver tissue specimen, and after keeping the specimen at room temperature for various periods of time. In  
10 Figure 2,

1 designates the infrared spectrum for the fresh, normal, healthy tissue specimen,

2 designates the infrared spectrum for the specimen kept at room temperature for one hour,

15 3 designates the infrared spectrum for the specimen kept at room temperature for 2 hours,

4 designates the infrared spectrum for the specimen kept at room temperature for 3 hours, and

20 5 designates the infrared spectrum for the specimen kept at room temperature for 4 hours.

Figure 2 shows that there is a decrease in infrared spectral absorption, the magnitude of which depends upon the length of time that the sample is kept at room temperature.

25 Figure 3 shows similar infrared spectra to that shown in Figure 2 but for normal healthy liver tissue, designated -----, and liver tissue found histologically to be malignant, designated ———, both of which were



immediately subjected to the infrared spectral absorption examination after bowel resection and without preservative treatment according to the present invention.

5 It will be seen by comparing Figures 2 and 3 that the spectral absorption intensity decreases in the range  $\sim 2960 \text{ cm}^{-1}$  and tends to become remarkably similar for the fresh, normal healthy tissue of Figure 2 compared to malignant tissue of Figure 3, the longer the normal healthy tissue of Figure 2 is kept at room temperature. In fact, the infrared spectra designated 5, Figure 2, is capable  
10 of misinterpretation for that of the malignant tissue, designated ———, shown in Figure 3.

Furthermore, other tissue anomalies will also be capable of misinterpretation in this manner with room temperature degraded, untreated,  
15 tissue or cells.

Figure 4 shows infrared spectra in the frequency range  $900$  to  $1500 \text{ cm}^{-1}$  obtained from an air dried specimen of fresh, exfoliated cervical cells which has been diagnosed as having high grade dysplasia and was not treated with a  
20 preservative.

In Figure 4, ——— designates the infrared spectrum for an air dried, fresh, exfoliated cervical cells, and ————— designates the infrared spectrum of the same sample after keeping it for three days at room temperature. It is evident  
25 from figure 4 that after the sample was keeping at room temperature for three days, the shape, frequencies and intensities of various absorption bands in the entire spectrum were changed dramatically.

Figure 5 shows infrared spectra in the frequency range 900 to 1500  $\text{cm}^{-1}$  obtained from a sample of preservative treated, fresh, normal, healthy, exfoliated cervical cells. The preservative treatment comprised immersing the sample in a physiologically balanced salt solution, removing excess solution in centrifuge, and then placing sample in the sample holder.

In Figure 5, ——— designates the infrared spectrum for a wet sample of preservative treated, fresh, normal, healthy, exfoliated cervical cells, and ----- designates the infrared spectrum of the same sample after it was air dried on an infrared optical window mounted on a sample cell. It is evident from Figure 5 that there is substantially no change in the infrared spectrum for the treated specimen after it was air dried at room temperature. Of particular interest is the fact that the infrared spectra for the treated specimen in both the wet and dry form are similar to that of untreated, normal healthy cells, see United States Patent No. 5,168,162, dated December 1, 1992, column 4, lines 45 to 57.

Figure 6 shows that infrared spectra that were obtained in the frequency range 900 to 1800  $\text{cm}^{-1}$  from a preservative treated and dried specimen of fresh, normal, healthy, exfoliated cervical cells, and after keeping the dried specimen for three months. The preservative treatment comprised immersing the sample in a 1% by weight, aqueous solution of sodium chloride, removing excess solution in centrifuge, and then placing sample in the sample holder. The spectra were taken after the sample was air dried at room temperature. It is evident from Figure 6 that there is substantially no change in spectral absorption for the treated specimen over the period of three months at room temperature.

Figure 7 shows infrared spectra in the frequency range 900 to 1800  $\text{cm}^{-1}$  obtained from a preservative treated specimen of fresh, exfoliated cervical cells

diagnosed as having low grade dysplasia (CIN 1), and after keeping the specimen for three months at room temperature. The cells were treated in the same manner as those described with reference to Figure 6. It is evident from Figure 7 that there is substantially no change in the spectra for the treated specimen over the period of three months at room temperature.

Figure 8 shows that infrared spectra that were obtained in the range 950 to 1500  $\text{cm}^{-1}$  from a preservative treated specimen of fresh, normal, healthy exfoliated cervical cells, and after keeping the specimen for 15 hours at room temperature. The preservative treatment comprised immersing the sample in a 10% by weight aqueous solution of formalin, removing excess solution in a centrifuge, and then placing the sample in the sample cell.

In Figure 8, ——— designates the infrared absorption for the fresh, normal, healthy cells, and ———— designates the infrared spectral absorption after keeping the sample at room temperature for 15 hours. It will be seen that there is substantially no change in spectral absorption for the treated specimen over the period of 15 hours. Of particular interest is the fact that the infrared spectra for the treated specimen are similar to that of untreated, normal, healthy cells, see United States Patent No. 5,168,162, dated December 1, 1992, column 4, lines 45 to 57.

Figure 9, ——— designates the infrared absorption in the frequency range 950 to 1500  $\text{cm}^{-1}$  obtained from a preservative treated specimen of fresh, exfoliated cervical cells diagnosed as having moderate dysplasia (CIN II), and ———— designates the infrared absorption after keeping the specimen for one and half months at room temperature. The preservative treatment comprised placing the wet, fresh sample on the surface of a crystalline potassium bromide window

mounted on a sample holder. The spectra were taken after the sample was air dried at room temperature. It is evident from Figure 9 that there is substantially no change in the spectra for the treated specimen over the period of one and half months at room temperature.

5

Figure 10 shows infrared spectra in the frequency range 950 to 1,500  $\text{cm}^{-1}$  obtained from two samples of preservative treated, fresh, biopsy of cervical tissue, and ----- designates treated, normal healthy tissue, and ----- designates tissue that has been diagnosed in a conventional, histological manner as malignant. The samples were preservative treated by immersing the sample in phosphate buffered saline, removing excess saline, and then placing the wet sample in the sample holder.

10

The most prominent absorption differences include the following findings in the malignant tissue:

15

- a) severe change in the intensity at bands near 970  $\text{cm}^{-1}$ , 1025  $\text{cm}^{-1}$ , 1047  $\text{cm}^{-1}$ , 1080  $\text{cm}^{-1}$ , 1160  $\text{cm}^{-1}$ , 1240  $\text{cm}^{-1}$ , 1400  $\text{cm}^{-1}$ , and 1460  $\text{cm}^{-1}$ , and
- b) significant shift at bands near 1080  $\text{cm}^{-1}$  and 1160  $\text{cm}^{-1}$ .

20

Figure 11 shows infrared spectra in the frequency range 950  $\text{cm}^{-1}$  to 1500  $\text{cm}^{-1}$  obtained from two samples of preservative treated, fresh exfoliated cervical cell specimens from fresh scrapings, and ----- designates normal healthy cells, and ----- designates cells diagnosed as inflammatory. The cells were treated in the same manner as those described with reference to Figure 5 in the wet state.

25

The most prominent absorption differences include the following findings in the inflammatory cells:

- a) severe change in intensity at bands near  $970\text{ cm}^{-1}$ ,  $1025\text{ cm}^{-1}$ ,  $1047\text{ cm}^{-1}$ ,  $1080\text{ cm}^{-1}$ ,  $1160\text{ cm}^{-1}$ ,  $1300\text{ cm}^{-1}$ ,  $1400\text{ cm}^{-1}$ , and  $1460\text{ cm}^{-1}$ .
- b) significant shift at bands near  $1080\text{ cm}^{-1}$  and  $1240\text{ cm}^{-1}$ .

Figure 12 shows infrared spectra in the frequency range  $900$  to  $1500\text{ cm}^{-1}$  obtained from two samples of preservative treated, dried specimens of fresh, exfoliated cervical cells, ----- designates normal healthy cells, and — designates cells diagnosed as having Candida infection. The cells were preservative treated in the manner as those described with reference to Figure 6.

The most prominent absorption differences include changes in the intensity in the Candida infected cells at bands near  $930\text{ cm}^{-1}$ ,  $1160\text{ cm}^{-1}$  and  $1240\text{ cm}^{-1}$  and in the broadening of the strong band near  $1025\text{ cm}^{-1}$ .

Figure 13 shows infrared spectra in the frequency range  $950$  to  $1,500\text{ cm}^{-1}$  obtained from two samples of preservative treated, fresh exfoliated cervical cells, ----- designates normal healthy cells, and — designates cells diagnosed as having dysplasia, CIN 1, HPV (human paparoma virus). The cells were preservative treated in the same manner as those described with reference to Figure 8.

The most prominent absorption differences include changes in the intensity in the dysplasia, CIN 1, HPV cells at bands near  $970\text{ cm}^{-1}$ ,  $1047\text{ cm}^{-1}$ ,  $1160\text{ cm}^{-1}$ ,  $1240\text{ cm}^{-1}$ ,  $1400\text{ cm}^{-1}$ , and  $1460\text{ cm}^{-1}$ .

In Figure 14, ——— designates the ATR infrared spectrum that were obtained in the frequency range 950 to 1500  $\text{cm}^{-1}$  from a preservative treated and dried specimen of cultured, malignant ovarian cells, and ----- designates the infrared spectrum after keeping the dried specimen for 20 hours. The cell were treated in the same manner as those described with reference to Figure 5. It is evident from Figure 14 that there is substantially no change in spectral absorption for the treated specimen over the period of three days at room temperature, except for a slight change on the baseline of the spectrum, which has no effect on the detection of the presence of anomalies.

Other preservatives may be used such as, for example, aqueous solutions of ethyl alcohol, methyl alcohol, ethyl ether, ethylene glycol or their mixtures, and it is also within the scope of the invention to use a preservative of other salines than phosphate buffered saline and physiologically balanced salt solution and of other inorganic salts than sodium chloride and potassium bromide. The preservative may be in aqueous solution or in solid state or of gaseous formaldehyde.

Tissue or cell anomalies which may be detected according to the present invention include, for example, precancerous lesions, viruses, bacteria, fungi and other infectious and non-infectious diseases, where infrared absorption occurs in the sample, at at least one range of frequencies, due to the vibration of at least one functional group of molecules being present in a sample which is characteristic of that tissue or cell anomaly. This can be determined by routine tests and the functional group of molecules detected may, for example, be from cellular carbohydrates, lipids, proteins, or nucleic acids.

Typical non-infectious diseases are cancer, diabetes, cirrhosis and arthritis.

5 Examples of the kinds of tissue or cells, which may be neoplastic, in which the presence of abnormality, e.g., malignancy, can be detected, according to the present invention, and for which tests have been carried out, include colorectal tumors (for detecting colon carcinoma), liver tumors (for detecting hepatomas), skin cancer, brain cancer, ovarian cancer, stomach cancer, esophagus cancer, endometrial cancer, cervical cancer, and other cancerous as  
10 well as neoplastic cells in blood.

15

20

25

WE CLAIM

1. A method of detecting the presence of anomalies, in a preservative treated sample therefor, prepared from fresh, biological tissue or cells in natural or cultured form including exfoliated cells, by infrared spectroscopy, comprising:

a) directing a beam of infrared light at the preservative treated sample, and

b) determining, by spectral analysis at least one range of frequencies, whether variation in infrared absorption occurs in the treated sample, distinguishable from any variation therein resulting from the treatment, due to vibration of at least one functional group of molecules that are present in the treated sample and characteristic of an anomaly.

2. A method according to claim 1, wherein the said determination by spectral analysis comprises comparing infrared spectra, obtained by directing the beam of infrared light at the treated sample, and spectral parameters derived therefrom, at the said at least one range of frequencies, with similar infrared spectra, and spectral parameters derived therefrom, from a similar, control sample, treated in the same manner, and known to have been prepared from fresh, normal, healthy tissue or cells.

3. A method according to claim 1, wherein the said determination by spectral analysis comprises, comparing infrared spectra obtained by directing the beam of infrared light at the treated sample, and spectral parameters derived from the spectra, at the said at least one range of frequencies, with similar



infrared spectra, and spectral parameters derived therefrom, from at least one similar sample, treated in the same manner, and having a known anomaly.

4. A method according to claim 1, wherein the said determination by  
5 spectral analysis is obtained from infrared radiation from the beam that has passed through the treated sample, at the said at least one range of frequencies.

5. A method according to claim 1, wherein the said determination by  
10 spectral analysis is obtained from attenuated, totally reflected, infrared radiation of the beam from the sample at the said at least one range of frequencies.

6. A method according to claim 1, wherein the preservative treated sample  
15 is a sample that has been treated with at least one preservative selected from the group consisting of formaldehyde, alcohols, salines and inorganic salts.

7. A method according to claim 6, wherein the formaldehyde is an aqueous  
solution of formalin.

8. A method according to claim 6, wherein the alcohol is an aqueous solution of  
20 ethyl alcohol, an aqueous solution of methyl alcohol and an aqueous solution of a mixture of ethyl alcohol and methyl alcohol.

9. A method according to claim 6, wherein the preservative is a saline  
25 selected from the group consisting of normal saline, phosphate buffered saline and a physiologically balanced salt solution.

10. A method according to claim 6, wherein the preservative is a substance selected from the group consisting of sodium nitrate, sodium chloride and potassium bromide.
- 5 11. A method according to Claims 1 and 6, wherein the preservative treated sample can be in either wet or dried form.
12. A method according to Claims 1 and 6, wherein the preservative treated sample comprises wet biological tissue or cells placed on the surface of  
10 crystalline inorganic salts as the solid preservative.
13. A method according to claim 1, wherein the said range of frequencies is at least one range of frequencies within the ranges  $500\text{ cm}^{-1}$  to  $900\text{ cm}^{-1}$ ,  $900\text{ cm}^{-1}$  to  $1500\text{ cm}^{-1}$ ,  $900\text{ cm}^{-1}$  to  $1800\text{ cm}^{-1}$  and  $2000\text{ cm}^{-1}$  to  $6000\text{ cm}^{-1}$ .

15

20

25

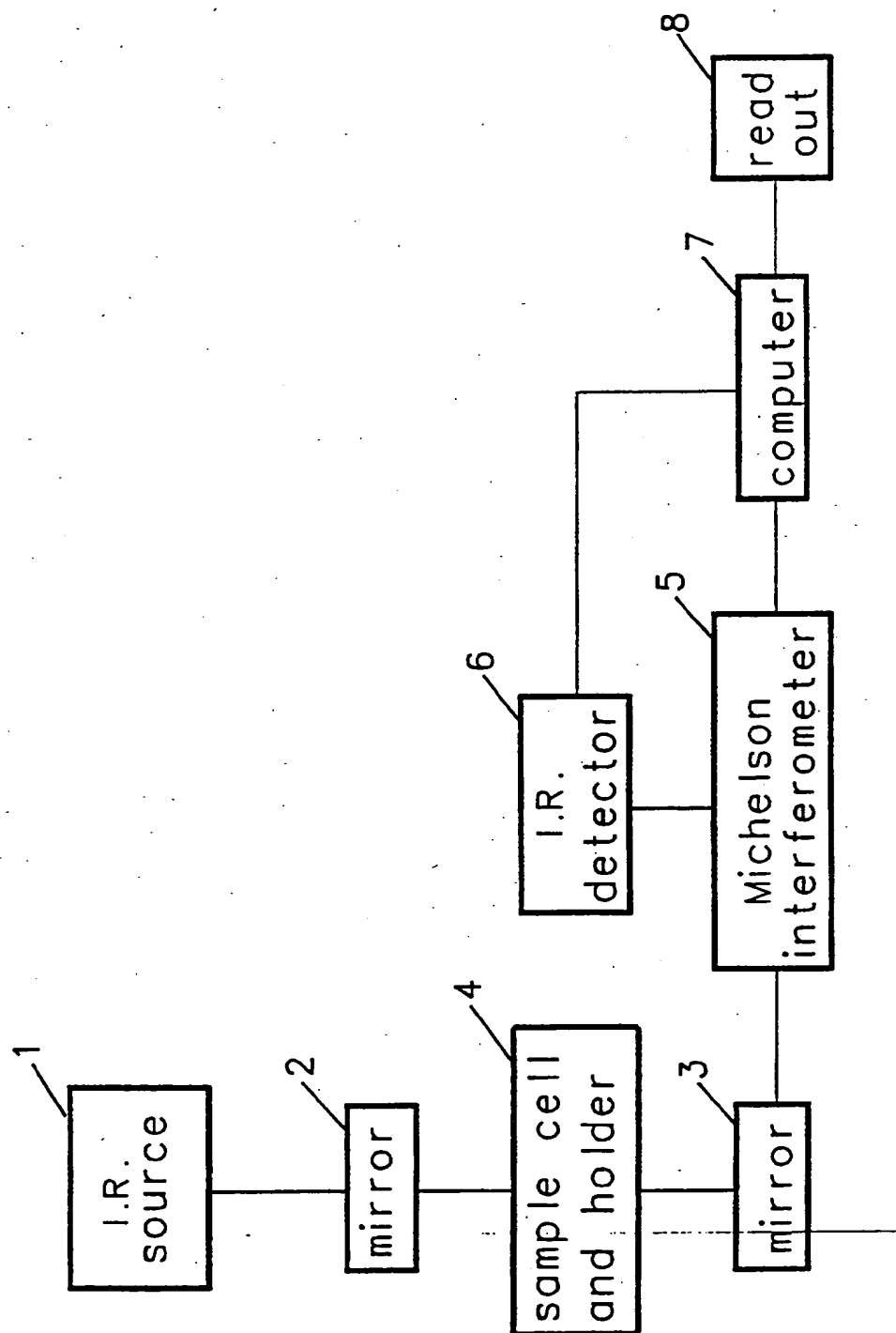


FIGURE 1

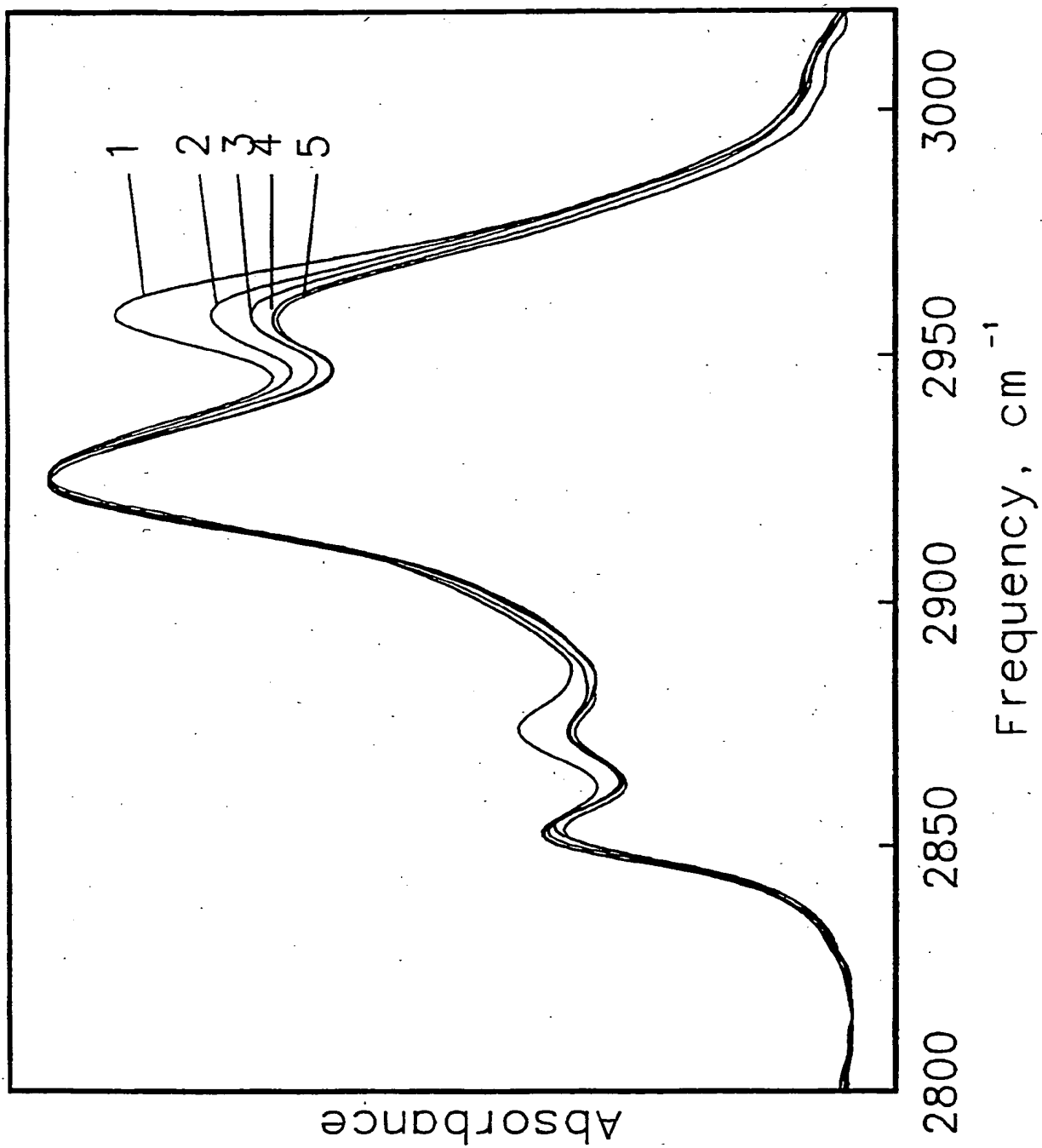


FIGURE 2

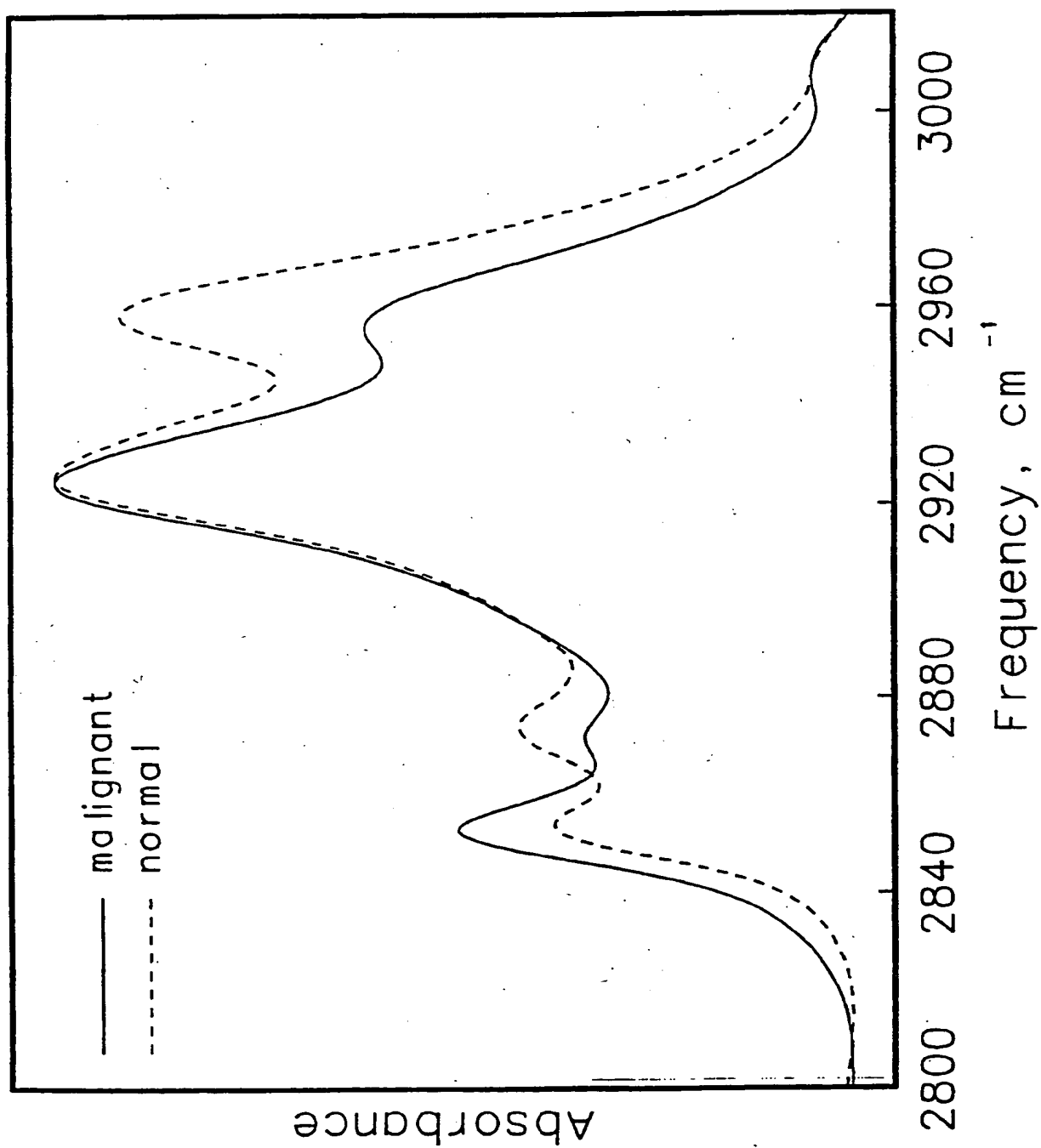


FIGURE 3

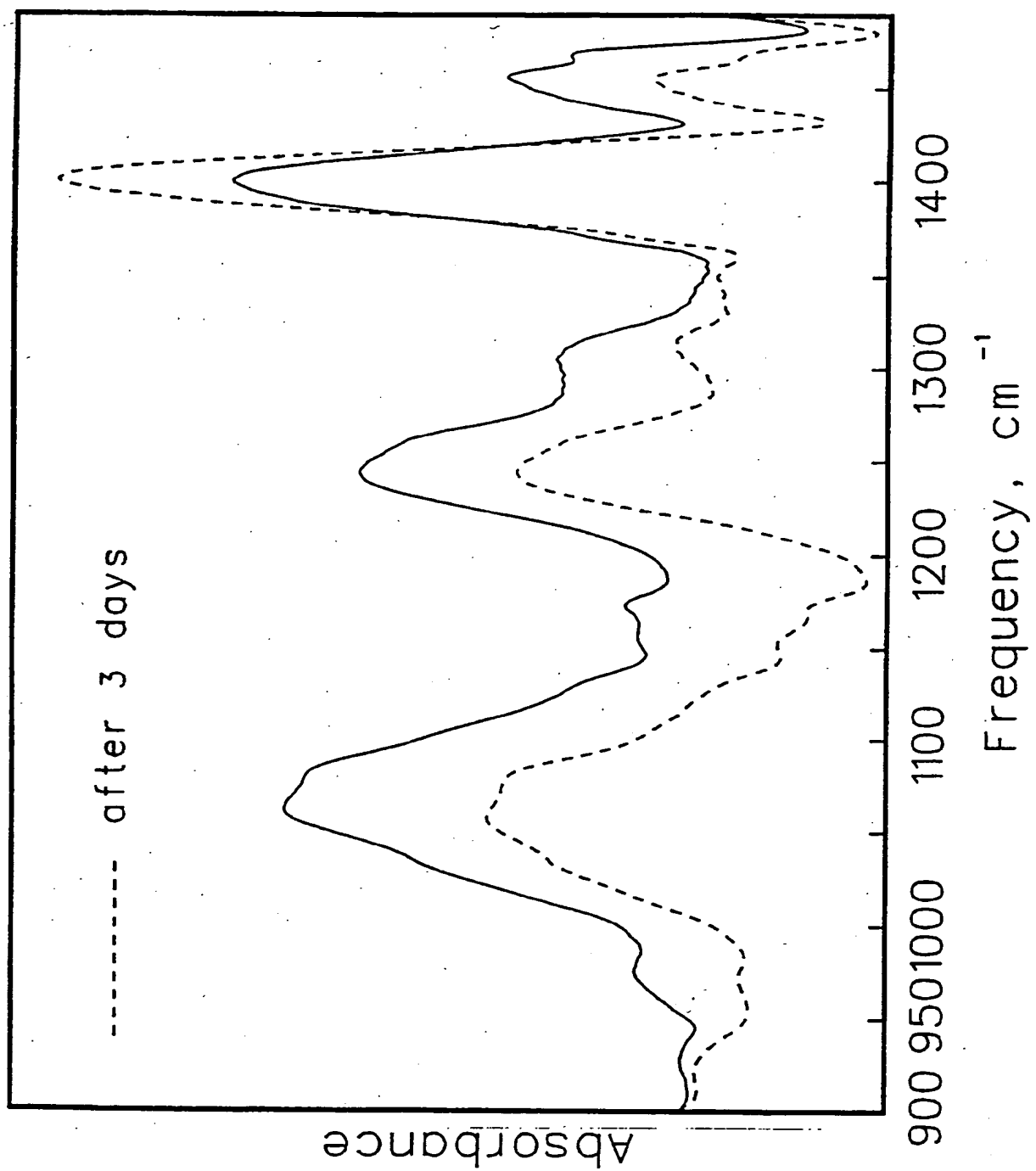


FIGURE 4

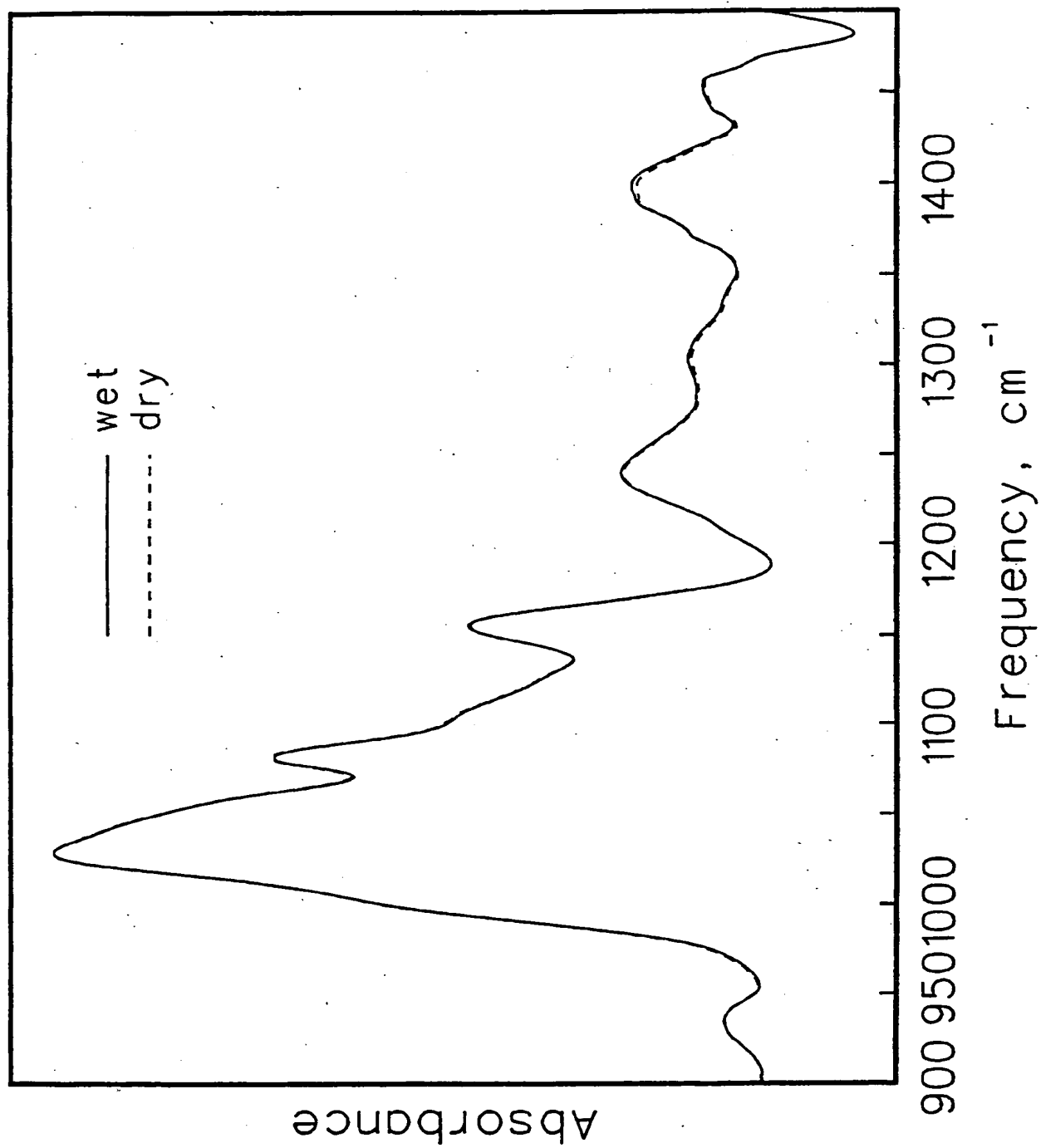


FIGURE 5

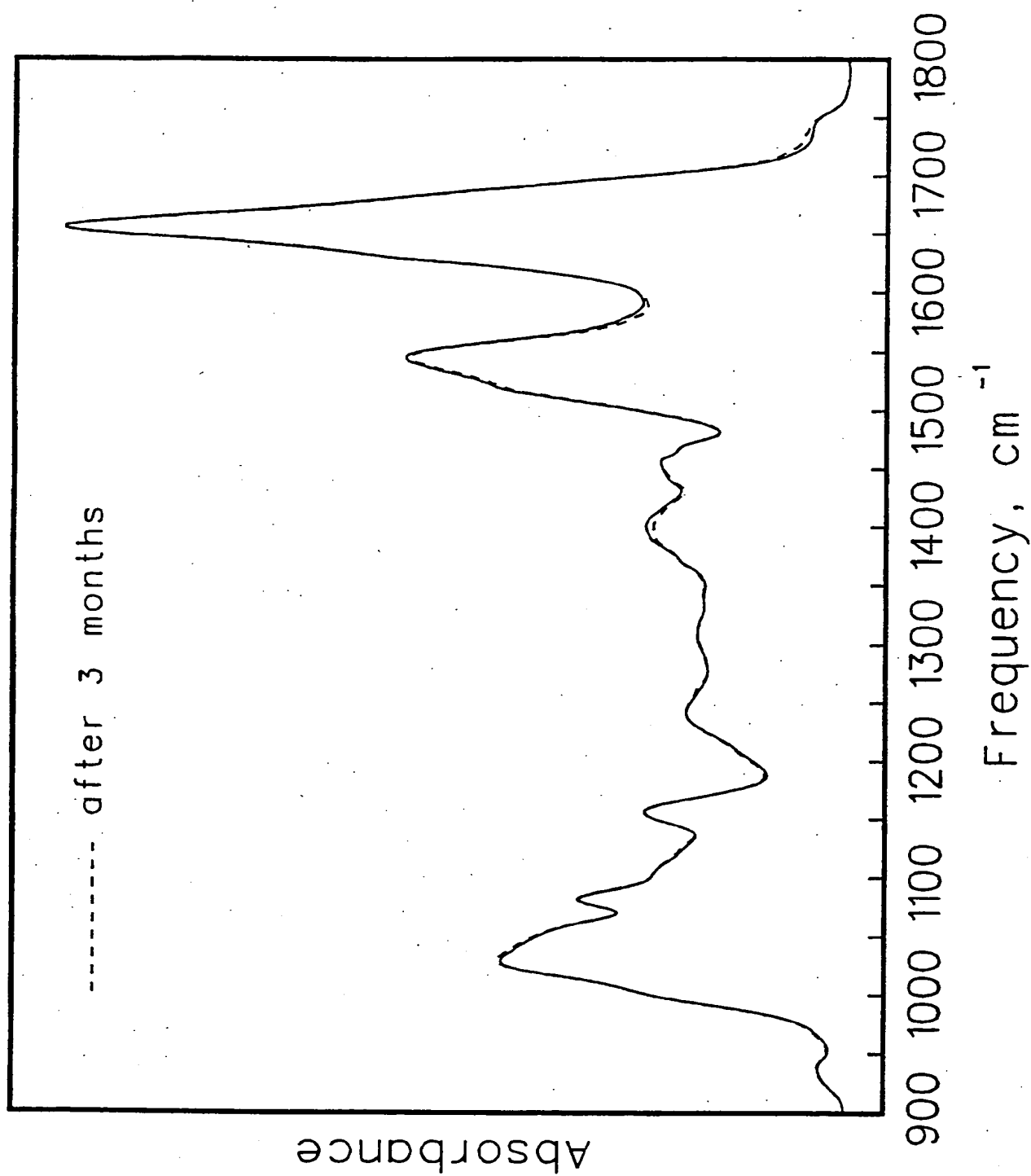


FIGURE 6



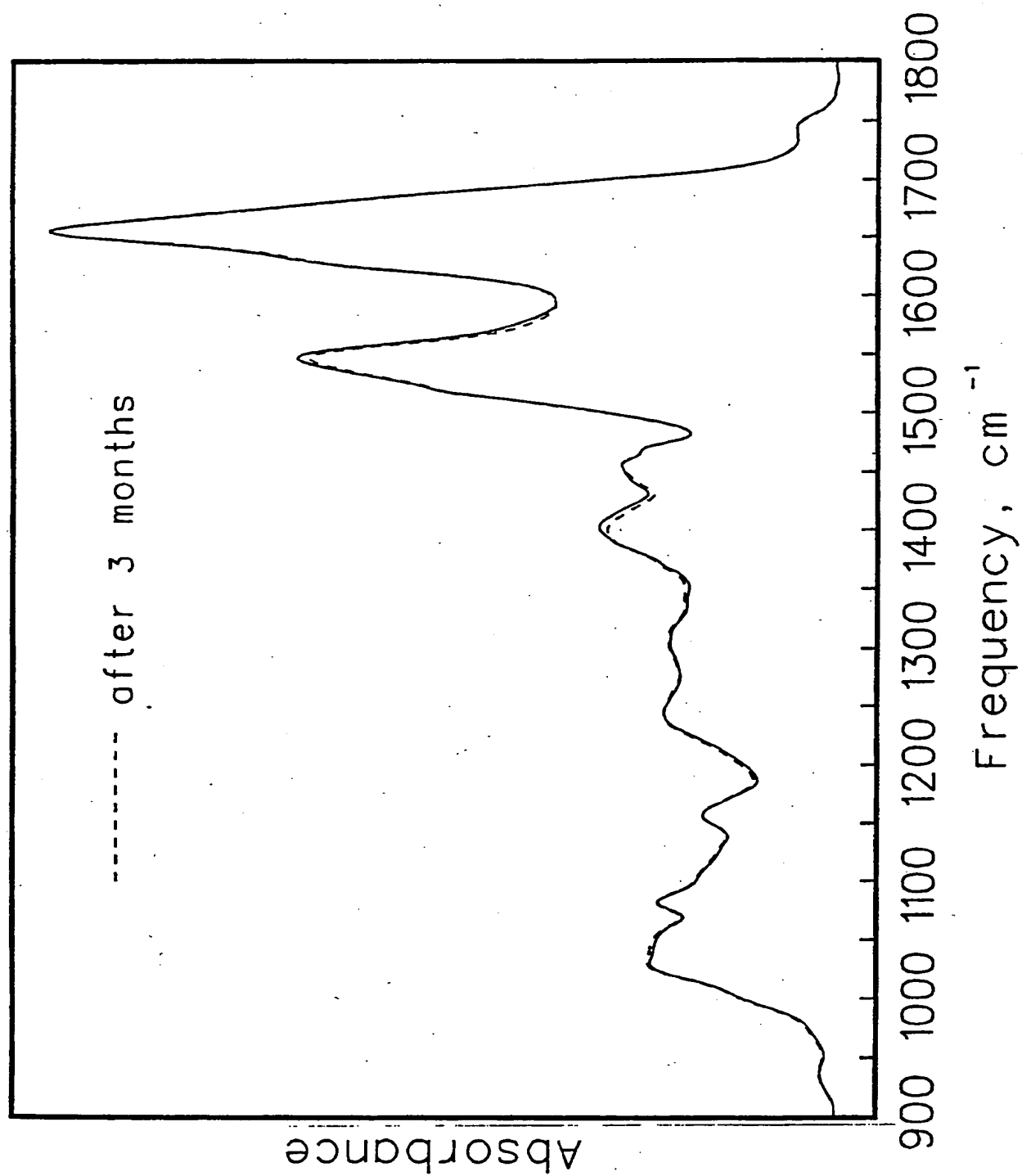


FIGURE 7

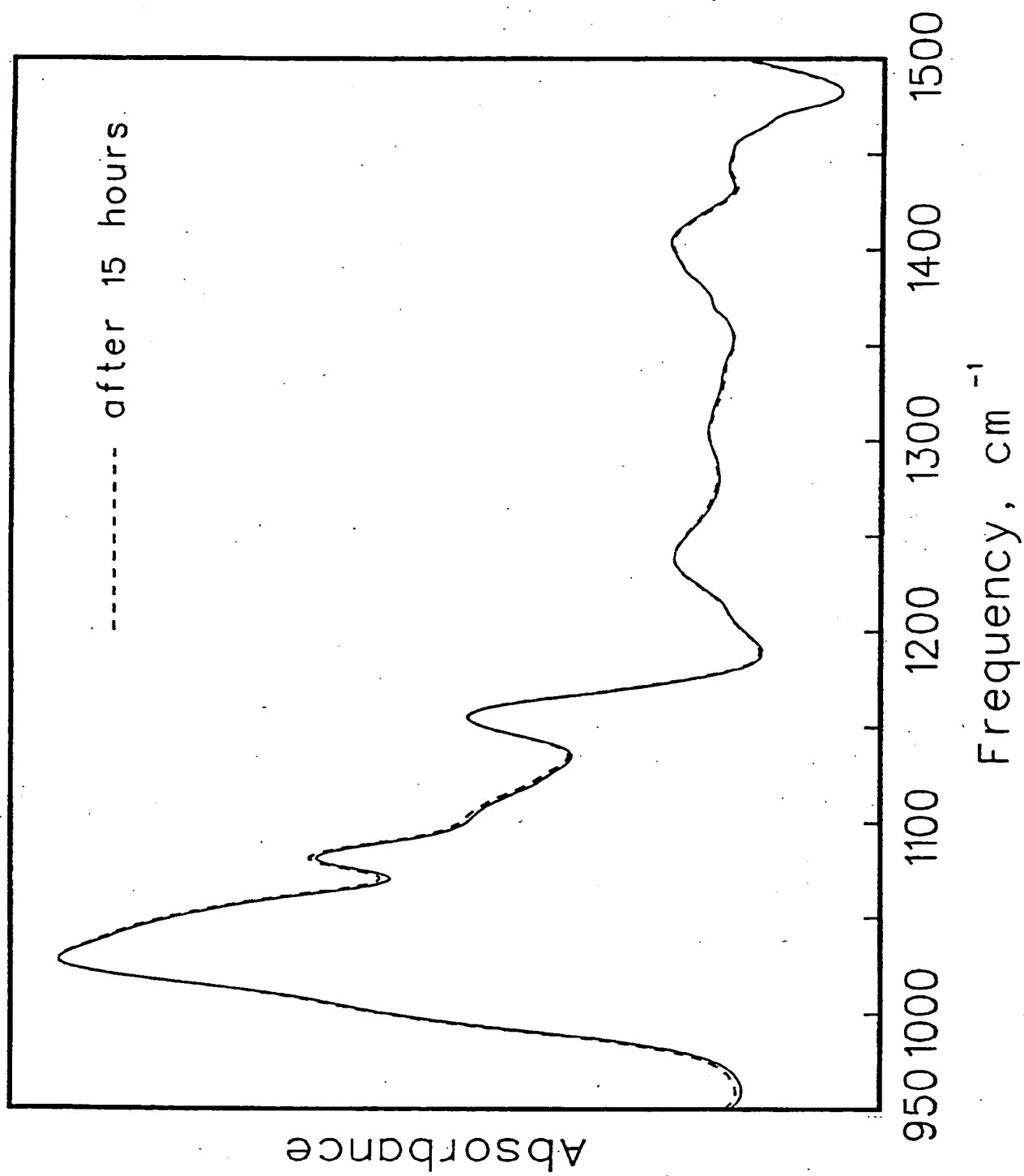


FIGURE 8

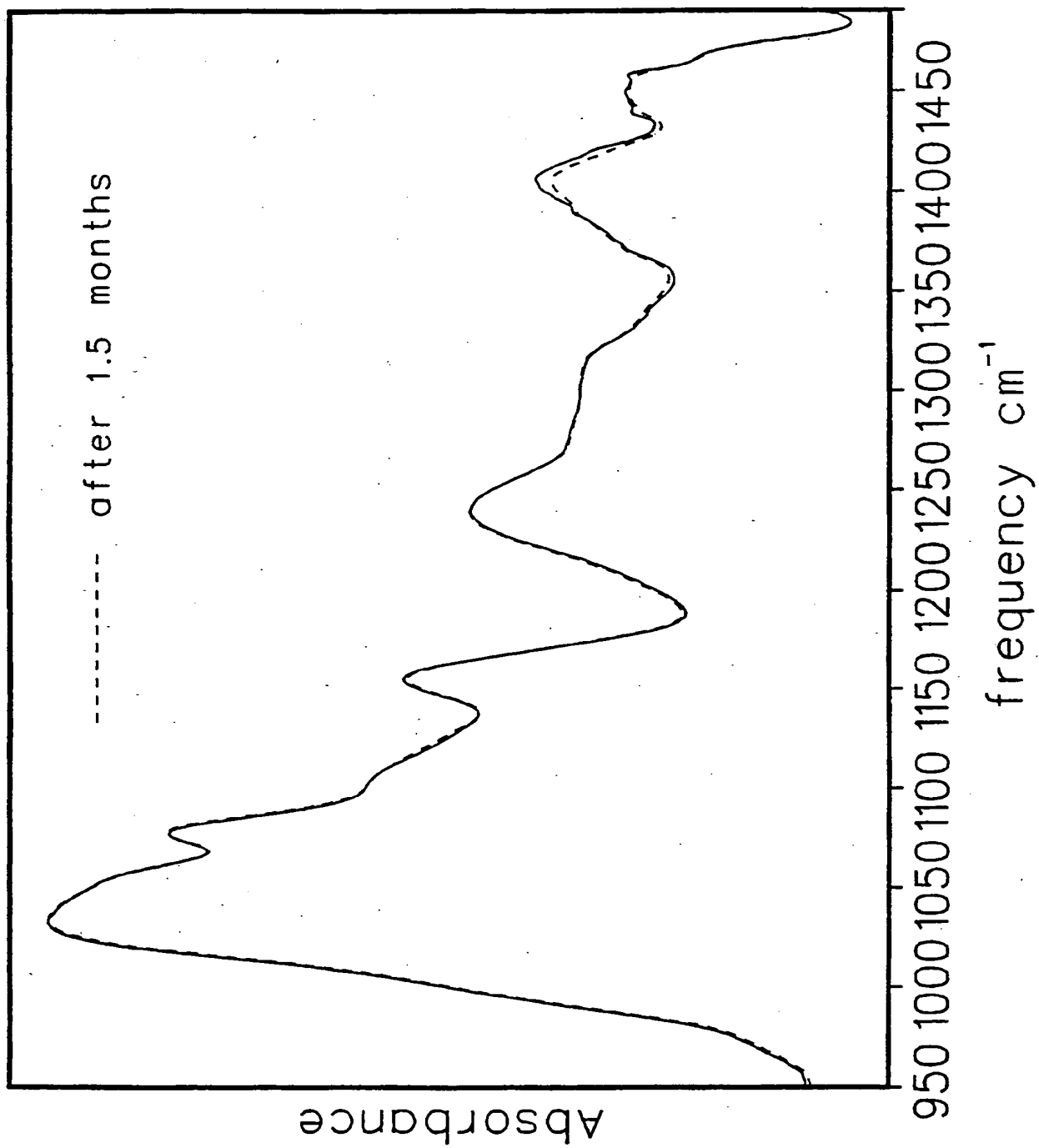


FIGURE 9

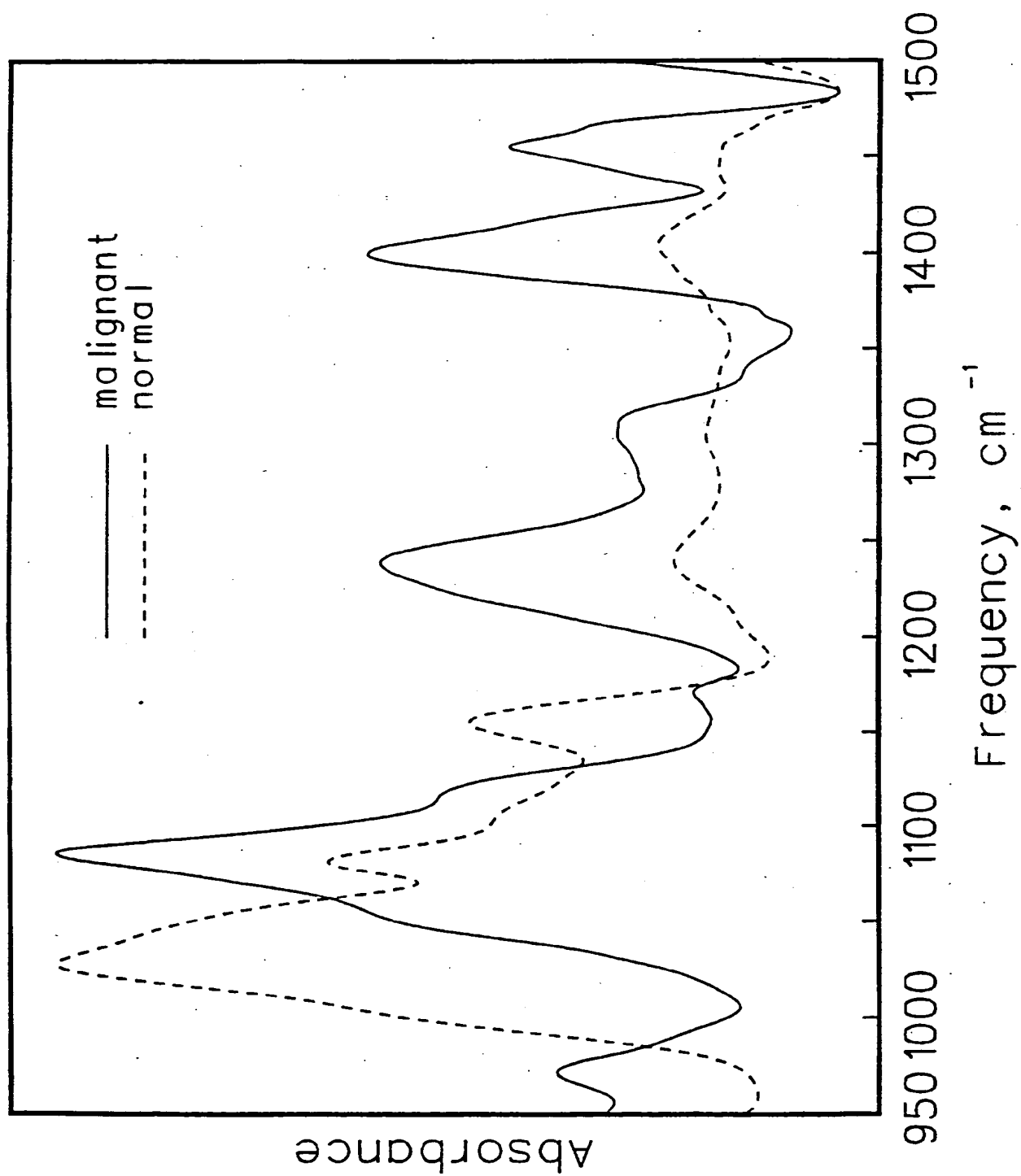


FIGURE 10

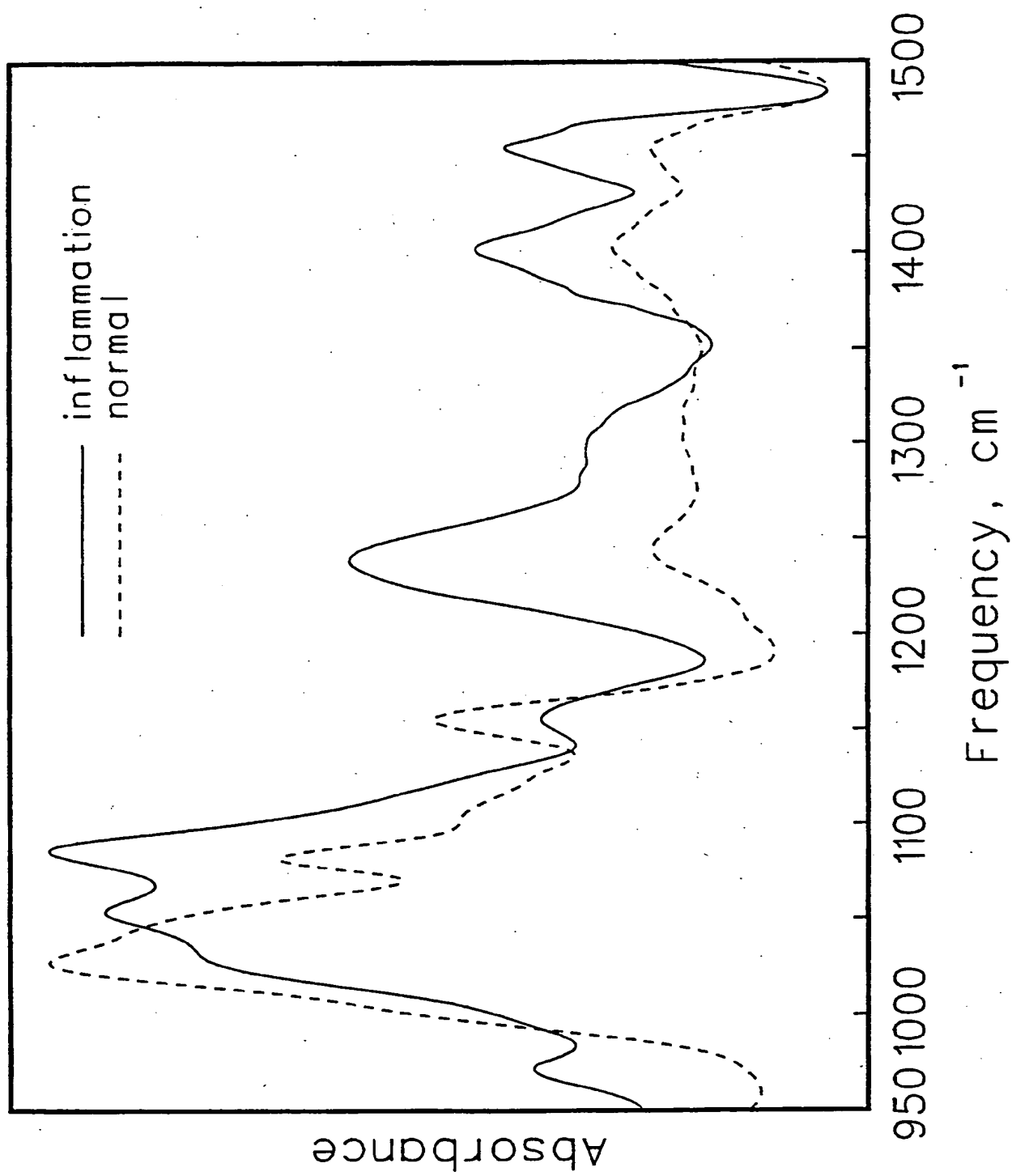


FIGURE 11

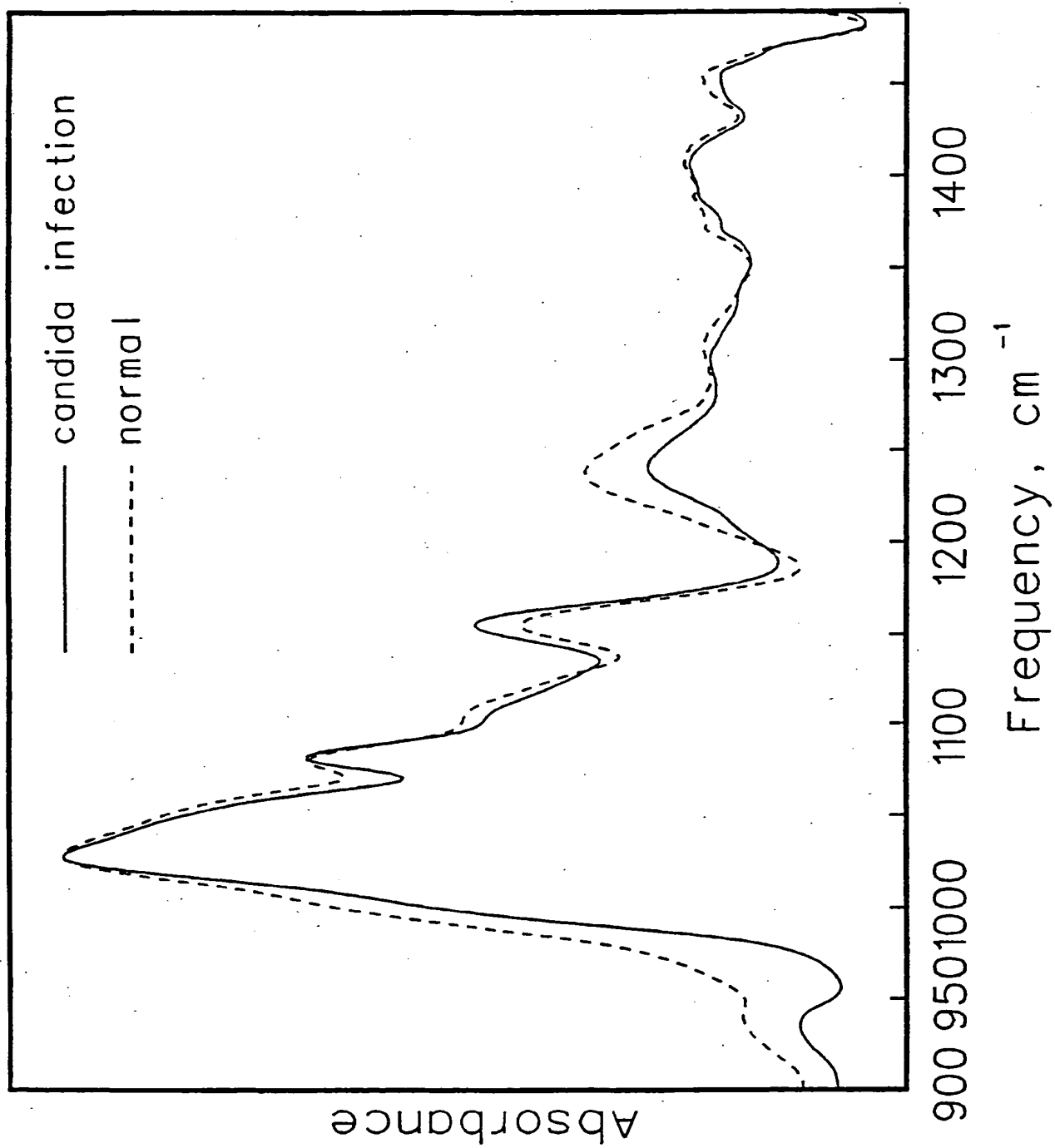


FIGURE 12

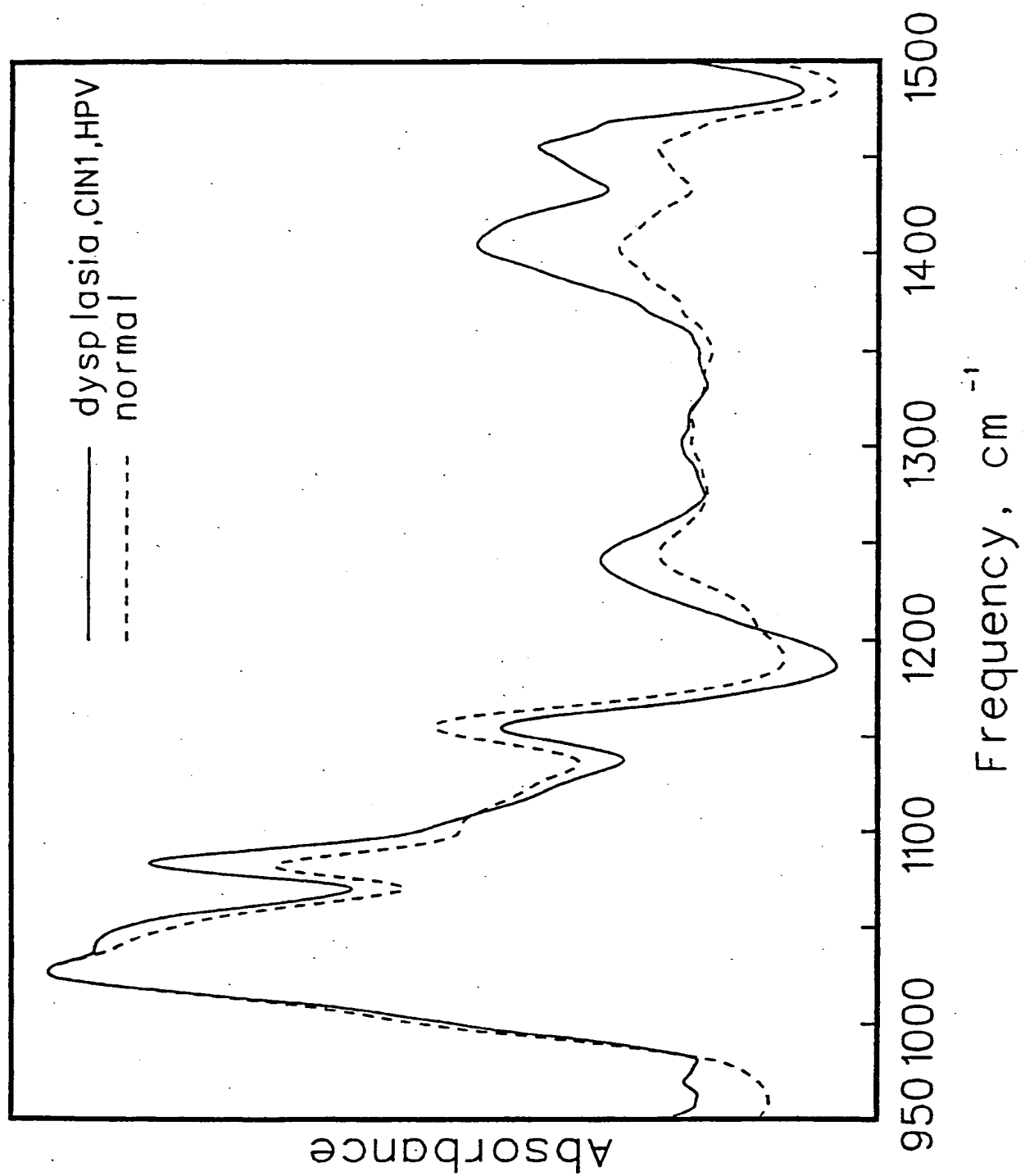


FIGURE 13

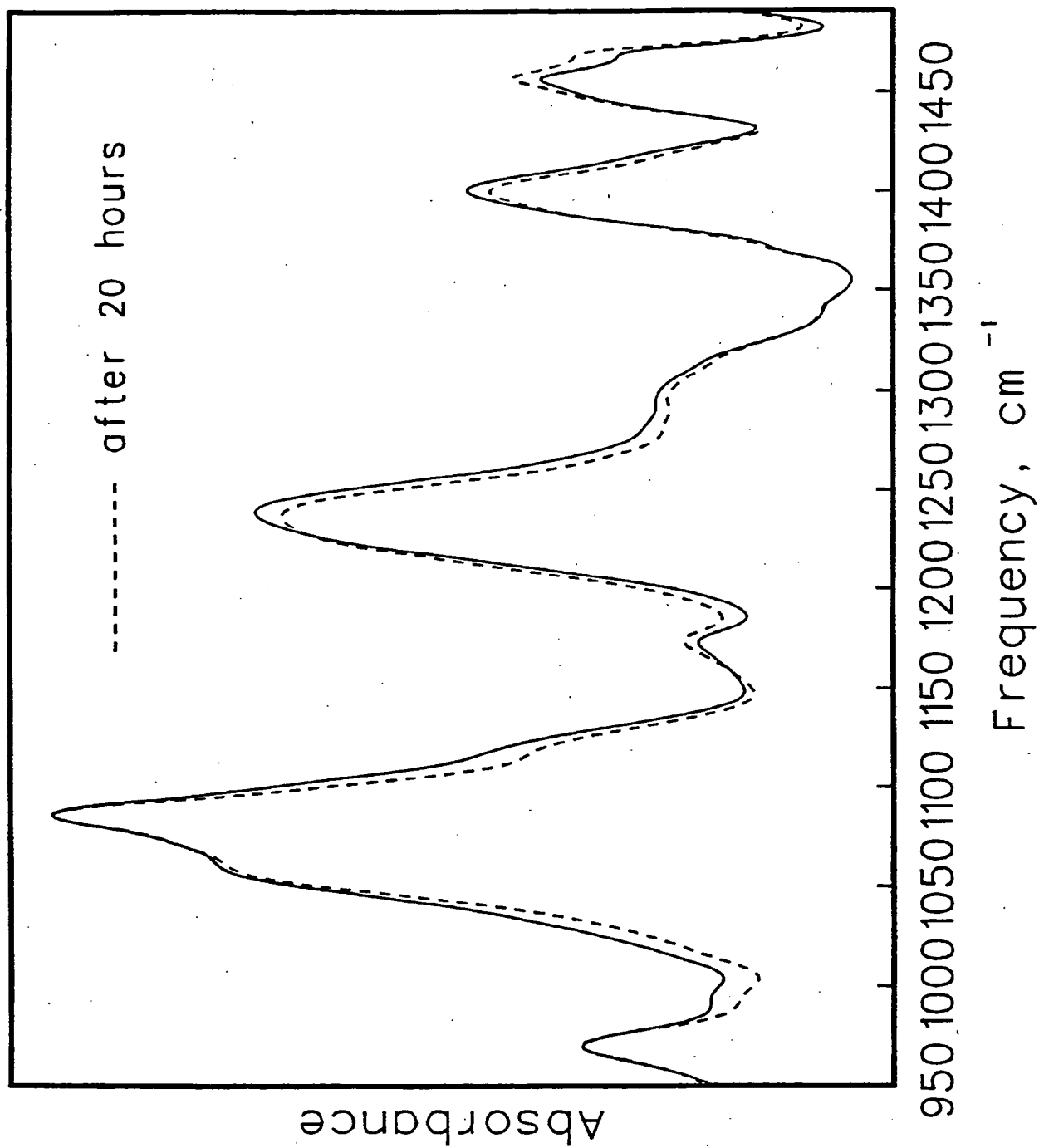


FIGURE 14



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 95/00370

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 G01N21/35 G01N33/483 C12M1/34

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANALYTICAL CHEMISTRY, vol. 66, no. 3, 1 February 1994 COLUMBUS US, pages 319-326, C.J. FRANK ET AL 'Characterization of human breast biopsy specimens with near-ir Raman spectroscopy' see page 319, left column, line 1 - right column, line 5 see page 320, right column, last paragraph - page 321, left column, line 14 see page 326, right column, line 17 - line 25	1,2,6,7, 13
A	US,A,5 168 162 (WONG) 1 December 1992 cited in the application see abstract see column 2, line 13 - line 20	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

Date of the actual completion of the international search

29 September 1995

Date of mailing of the international search report

11 10. 95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Thomas, R.M.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 95/00370

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5168162	01-12-92	CA-A- 2035603	05-08-92
		AU-B- 1180892	07-09-92
		WO-A- 9214134	20-08-92
		CN-A- 1067314	23-12-92
		EP-A- 0570418	24-11-93
		JP-T- 6507237	11-08-94
<hr/>			